VERIFICATION OF TRANSLATION

I, Takayoshi Hirose residing at Takahashi Bldg. Kita-sangokan, 13-3, Nishitenma 5-chome, Kita-ku, Osaka-shi, Osaka 530-0047 Japan, hereby declare that:

1. I read and understand the Japanese and English languages.

2. I translated the International application PCT/JP2004/016776 from Japanese into English.

3. The annexed document is, to the best of my knowledge and belief, an accurate translation of the International application PCT/JP2004/016776.

This 28th day of April, 2006
Osaka, Japan

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SPECIFICATION

HLA-E CHIMERIC MOLECULE

TECHNICAL FIELD

The present invention relates to HLA-E chimeric molecule. More particularly, it relates to HLA-E chimeric molecules for providing nonhuman mammal cell with resistance to cytotoxicity by human NK cell, base sequences for coding the chimeric molecules, and nonhuman mammal cell and nonhuman mammal animal transformed by the base sequences.

BACKGROUND ART

Organ transplantation is a very useful therapeutic method. Organ transplantation is classified into allotransplantation and xenotransplantation, which have their own merits and demerits. The human-to-human allotransplantation is an established method, but it is limited in the number of donors. Xenotransplantation from nonhuman mammal (for example, swine) to human is multiple in selection of grafts, but it is always accompanied by specific rejections such as hypoacute rejection (HAR) and acute vascular rejection (AVR).

To overcome such specific rejections of xenotransplantation, various methods have been proposed, such as a method of expressing human complement inhibitor in nonhuman mammals (for example, patent document 1), a method of decreasing Galα1,3Gal sequences (hereinafter referred to as α-Gal antigen) of non-reducing terminals of sugar chains not existing in primates or humans but existing in nonhuman mammals (for example, patent document 2), and a method of knocking out genes of α1,3

galactosyl transferase responsible for generation of α-Gal antigen (for example, non-patent documents 1 and 2).

[Patent document 1] Japanese unexamined patent publication No. H11-239430

[Patent document 2] Japanese unexamined patent publication No. 2002-291372

[Non-patent document 1] Science 2002, 295, 1089

[Non-patent document 2] Nat. Biotechnol. 2002, 20, 251

When a graft of nonhuman mammal is transplanted on human recipient, if the HAR can be overcome, the latter's antibody (anti-α-Gal antibody, etc.), complement, platelet, or natural killer (NK) cell may adhere to the former's cell, and the cell may be activated. The activated cell releases various cytokines, dissociates heparin, produces gaps against adjacent cells, exposes collagen of basement membrane, induces blood clotting reaction, closes blood vessels, and necrotizes grafts of nonhuman mammal (non-patent document 3). Such rejections are known as acute vascular rejections (AVR), and so far no method has been known to suppress efficiently the cytotoxicity of NK cell known as one of AVR factors.

[Non-patent document 3] Xenotransplantation 1998, 5, 169

NK cell adheres target cells by way of two types of receptors. They are killer cell activating receptor for inducing cytotoxicity, and killer cell suppressing receptor for suppressing cytotoxicity by recognizing the own MHC class I molecule. When the signal from the former surpasses the signal from the latter, the target cell is necrotized, but when the signal from the latter surpasses the signal from the former, the target cell is not necrotized.

The human cell expresses HLA class I molecules (HLA-A, -B, -C, -E, -F,

-G), and hence the human cell is not damaged by human NK cell. On the other hand, sine the nonhuman mammal cell does not express HLA class I molecules, it is damaged by human NK cell. Hence, a new method has been developed to avoid cell damage by human NK cell by transforming the nonhuman mammal cell by genes of human HLA-A, HLA-B, HLA-C, or HLA-G (patent document 3). However, HLA-A, HLA-B, HLA-C are polymorphic, having 175, 344, and 90 alleles respectively, and it is not practical to prepare nonhuman mammal cells applicable to each HLA.

On the other hand, HLA-E and HLA-G are not polymorphic, and it has been attempted to use HLA-E and HLA-G. As a result, it is relatively easy to express HLA-G on the surface of nonhuman mammal cell, but suppression of cytotoxicity by human NK cell is low, and to the contrary it is found not easy to express HLA-E on the surface of nonhuman mammal cell, but suppression of cytotoxicity by human NK cell is high (non-patent document 4).

For the purpose of increasing the HLA-E expressing amount on nonhuman mammal cell surface, it has been attempted to use base sequence for coding the HLA-E, β_2 microglobulin and HLA-A2 leader peptide (Val-Met-Ala-Pro-Arg-Thr-Leu-Val-Leu), or base sequence for coding the leader peptide of HLA-G (Val-Met-Ala-Pro-Arg-Thr-Leu-Phe-Leu). However, the HLA-E expressing amount by these transformants and the suppression of cytotoxicity by NK cell were not sufficient (non-patent document 5).

[Patent document 3] Japanese unexamined patent publication No. H11-510698

[Non-patent document 4] Transplantation Proceedings 2000, 32, 939

[Non-patent document 5] Transplantation 2002, 73, 1582

DISCLOSURE OF THE INVENTION

The invention is devised to solve the problems of the prior art, and the inventors have been intensively studied about HLA-E chimeric molecule for providing the nonhuman mammal cell with resistance to cytotoxicity by human NK cell, and prepared base sequences for coding

(1) HLA-E chimeric molecule replacing all or part of α2 domain of HLA-E molecule with all or part of α2 domain of HLA-G1 molecule,

(2) HLA-E chimeric molecule replacing, together with (1), signal peptide (SP) of HLA-E molecule with reformed SP partly reforming the SP of HLA-G1 molecule, or

(3) HLA-E chimeric molecule replacing, together with (2), part of amino acid sequence of α1 domain and α2 domain of HLA-E molecule, with part of amino acid sequence of α1 domain and α2 domain of HLA-G1 molecule, respectively, and

attempted to transform the nonhuman mammal cell by using them, and found that the expressing amount of HLA-E chimeric molecules is increased and that the resistance to cytotoxicity by human NK cell is increased, thereby completing the invention.

That is, the invention presents HLA-E chimeric molecules for providing nonhuman mammal cell with resistance to cytotoxicity by human NK cell, base sequence for coding them, and nonhuman mammal cells and nonhuman mammal animals transformed by the base sequences.

The reformed SP means a sequence of amino acid sequence of SP in which one or two or more amino acids are replaced or deleted, or one or more amino acids are added, and examples include SP (Met Ala Val Met Ala Pro Arg Thr Leu Val Leu Leu Leu Ser Gly Ala Leu Thr Leu Thr Glu Thr Trp

Ala: sequence number 21, hereinafter referred to as reformed SP) by reforming SP (sequence number 11) of HLA-G1 molecule.

Amino acid sequences of signal peptide (SP) of HLA-E molecule, α1 domain, α2 domain, α3 domain and transmembrane (TM) domain are respectively shown in sequence numbers 1 to 5, and their base sequences are shown in sequence numbers 6 to 10.

Amino acid sequences of SP of HLA-G1 molecule, α1 domain, α2 domain, α3 domain and TM domain are respectively shown in sequence numbers 11 to 15, and their base sequences are shown in sequence numbers 16 to 20.

BEST MODE FOR CARRYING OUT THE INVENTION

To achieve the object, the invention presents HLA-E chimeric molecules for providing nonhuman mammal cell with resistance to cytotoxicity by human NK cell, and base sequence for coding them, and more specific examples are chimeric molecules having the following properties and base sequences for coding them.

(1) HLA-E chimeric molecule having reformed SP, being HLA-E chimeric molecule except that amino acid number 91-182 (number from α1 domain N terminal, same hereinafter) of α2 domain of HLA-E molecule is replaced by amino acid number 91-182 of α2 domain of HLA-G1 molecule and a base sequence for coding it. In this chimeric molecule, amino acid sequences of SP, α1 domain, α2 domain, α3 domain, and TM domain are respectively shown in sequence numbers 21 to 25, and their base sequences are respectively shown in sequence numbers 26 to 30;

(2) HLA-E chimeric molecule having reformed SP, being HLA-E chimeric molecule except that amino acid number 137-182 of α2 domain

latter part of HLA-E molecule is replaced by amino acid number 137-182 of α2 domain latter part of HLA-G1 molecule and a base sequence for coding it. In this chimeric molecule, amino acid sequences of SP, α1 domain, α2 domain, α3 domain, and TM domain are respectively shown in sequence numbers 31 to 35, and their base sequences are respectively shown in sequence numbers 36 to 40;

(3) HLA-E chimeric molecule having reformed SP, being HLA-E chimeric molecule except that fore part amino acid number 137-150 of α2 domain latter part of HLA-E molecule is replaced by fore part amino acid number 137-150 of α2 domain latter part of HLA-G1 molecule and a base sequence for coding it. In this chimeric molecule, amino acid sequences of SP, α1 domain, α2 domain, α3 domain, and TM domain are respectively shown in sequence numbers 41 to 45, and their base sequences are respectively shown in sequence numbers 46 to 50;

(4) HLA-E chimeric molecule having SP of HLA-E molecule or reformed SP, being HLA-E chimeric molecule except that amino acid number 147 of α2 domain of HLA-E molecule is replaced by cysteine and a base sequence for coding it. In this chimeric molecule, amino acid sequences of SP, α1 domain, α2 domain, α3 domain, and TM domain of chimeric molecule having SP of HLA-E molecule are respectively shown in sequence numbers 51 to 55, their base sequences are respectively shown in sequence numbers 56 to 60, and amino acid sequences of SP, α1 domain, α2 domain, α3 domain, and TM domain of chimeric molecule having reformed SP are respectively shown in sequence numbers 61 to 65, and their base sequences are respectively shown in sequence numbers 66 to 70;

(5) HLA-E chimeric molecule having SP of HLA-E molecule or reformed SP, being HLA-E chimeric molecule except that amino acid

number 11 of α1 domain of HLA-E molecule is replaced by alanine, and that amino acid number 147 of α2 domain is replaced by cysteine and a base sequence for coding it. In this chimeric molecule, amino acid sequences of SP, α1 domain, α2 domain, α3 domain, and TM domain of chimeric molecule having SP of HLA-E molecule are respectively shown in sequence numbers 71 to 75, their base sequences are respectively shown in sequence numbers 76 to 80, and amino acid sequences of SP, α1 domain, α2 domain, α3 domain, and TM domain of chimeric molecule having reformed SP are respectively shown in sequence numbers 81 to 85, and their base sequences are respectively shown in sequence numbers 86 to 90; and

(6) Nonhuman mammal cell or nonhuman mammal animal provided with resistance to cytotoxicity by human NK cell, prepared and transformed by one base sequence for coding the HLA-E chimeric molecule in any one of (1) to (5) mentioned above.

The human HLA class I molecule consists of signal peptide (SP), α1 domain, α2 domain, α3 domain, and transmembrane (TM) domain, and further includes β_2 microglobulin (β_2m).

The human HLA class I molecule also presents antigenicity by sterically incorporating oligopeptide derived from signal peptide (SP) into the groove formed by α1 domain and α2 domain.

As mention above, it is relatively easy to transform the nonhuman mammal cell by using HLA-G gene, but a suppressing capacity of damage by human NK cell is low. To the contrary, a higher suppressing capacity of damage by human cell NK cell is obtained by transforming the nonhuman mammal cell by using HLA-E gene, but it is not easy to transform. Accordingly, in order to provide the nonhuman mammal cell with resistance to cytotoxicity by human NK cell, it has been attempted to prepare a base

sequence for coding the HLA-E chimeric molecule by exchanging domains in the HLA-E molecule and HLA-G1 molecule, transform the cell line of nonhuman mammals, and analyze increase or decrease of expressing intensity by FACS, by using anti-HLA antibody (B9.12.1 Cosmobio). As a result, as shown in Examples below, it is found that the HLA-E expression amount of nonhuman mammal cell is increased when transformed by the base sequence for coding the following HLA-E chimeric molecules, and that the resistance to cytotoxicity by human NK cell is increased.

(1) HLA-E chimeric molecule (see sequence numbers 21 to 30) prepared by replacing SP (sequence number 1) of HLA-E molecule with reformed SP (sequence number 21; reformed SP used in Example below), and replacing α2 domain (amino acid number 91-182) of HLA-E molecule with α2 domain (amino acid number 91-182) of HLA-G1 molecule,

(2) HLA-E chimeric molecule (see sequence numbers 31 to 40) prepared by replacing SP of HLA-E molecule with the reformed SP, and replacing latter part (amino acid number 137-182) of α2 domain of HLA-E molecule with latter part (amino acid number 137-182) of α2 domain of HLA-G1 molecule,

(3) HLA-E chimeric molecule (see sequence numbers 41 to 50) prepared by replacing SP of HLA-E molecule with the reformed SP, and replacing fore part (amino acid number 137-150) of latter part of α2 domain of HLA-E molecule with fore part (amino acid number 137-150) of latter part of α2 domain of HLA-G1 molecule,

(4) HLA-E chimeric molecule (see sequence numbers 51 to 60 and sequence numbers 61 to 70) prepared by replacing or not replacing SP of HLA-E molecule with the reformed SP, and replacing serine of amino acid number 147 of α2 domain of HLA-E molecule with cysteine of amino acid

number 147 of α2 domain of HLA-G1 molecule, and

(5) HLA-E chimeric molecule (see sequence numbers 71 to 80 and sequence numbers 81 to 90) prepared by replacing or not replacing SP of HLA-E molecule with the reformed SP, and replacing serine of amino acid number 11 of α1 domain of HLA-E molecule and serine of amino acid number 147 of α2 domain of the same with alanine of amino acid number 11 of α1 of HLA-G1 molecule and cysteine of amino acid number 147 of α2 of the same.

By building up a transgene by using a base sequence for coding one of the HLA-E chimeric molecules of (1) to (5) mentioned above, and gene promoter (for example, β-actin promoter, pMCP promoter, etc.), and/or other expression adjusting sequence, and transforming the nonhuman mammal cell by using the transgenes, it is possible to prepare nonhuman mammal cell having resistance to cytotoxicity by human NK cell.

By injecting the transgene into a fertilized egg of nonhuman mammal by microinjection method, it is possible to prepare nonhuman transgenic mammals composed of cells, tissues and organs having resistance to cytotoxicity by human NK cell. In the invention, nonhuman transgenic mammals are not particularly specified as far as nonhuman, and examples include swine, mouse, rat, hamster, cow, horse, sheep, rabbit, dog and cat, and considering xenotransplantation, swine may be preferred as a donor.

Further, by applying the nuclear-transfer method using the nonhuman mammal cell having resistance to cytotoxicity by human NK cell as donor cells, it is possible to prepare nonhuman cloned mammals composed of cells, tissues and organs having resistance to cytotoxicity by human NK cell. These nonhuman transgenic mammals or nonhuman cloned mammals can be prepared by properly selecting from the known methods and conditions.

INDUSTRIAL APPLICABILITY

The HLA-E chimeric molecule of the invention can be expressed efficiently on nonhuman mammal cells, and the resistance to cytotoxicity by human NK cell can be applied to nonhuman mammal cells. Therefore, the HLA-E chimeric molecule of the invention can effectively prevent generation of cell damage or acute vascular rejection (AVR) by human NK cell caused at the time of xenotransplantation of cells, tissues and organs of nonhuman mammals on human recipients.

EXAMPLES

The invention is more specifically described below by referring to Examples. It must be noted however that the invention is not limited to these Examples alone.

Example 1

Expression of various HLA-E chimeric molecules in nonhuman mammal cell (1)

A base sequence for coding the amino acid sequence composed as shown in Table 1 was incorporated into an expression vector of pCXN (β-actin promoter of chicken, having enhancer of CMV). Each transgene was incorporated into CHO cell, and the relative value of expression amount was determined by FACS analysis by using anti-HLA antibody (Pan-Class I antibody, B9.12.1 Cosmobio). Preparation of expression vector and operation of transfection conformed to the ordinary method of gene recombinant technology (same hereinafter).

Results are shown in Table 1.

Table 1. Expression of various HLA-E chimeric molecules in nonhuman mammal cell

Symbol	Molecule composition	Expression (relative amount)
(1) HLA-G1	HLA-G1 molecule	100
(2) HLA-E	HLA-E molecule	<1
(3) HLA-E(V)	Replaced SP of above (2) by reformed SP	2
(4) E(V)-TM	Replaced TM of above (3) by TM of above (1)	2
(5) E(V)-α3TM	Replaced α3, TM of above (3) by α3, TM of above (1)	<1
(6) E(V)-α2	Replaced α2 of above (3) by α2 of above (1)	62
(7) E(V)-α1α3TM	Replaced α1, α3, and TM of above (3) by α1, α3, and TM of above (1)	<1
(8) E(V)-α1	Replaced α1 of above (3) by α1 of above (1)	<1
(9) E(V)-α1-1	Replaced fore part of α1 of above (3) by fore part of α1 of above (1)	<1
(10) E(V)-α1-2	Replaced latter part of α1 of above (3) by latter part of α1 of above (1)	<1
(11) E(V)-α2-1	Replaced fore part of α2 of above (3) by fore part of α2 of above (1)	<1
(12) E(V)-α2-2	Replaced latter part of α2 of above (3) by latter part of α2 of above (1)	29
(13) E(V)-α2-2-1	Replaced fore part of latter part of α2 of above (3) by fore part of latter part of α2 of above (1)	26
(14) E(V)-α2-2-2	Replaced hind part of latter part of α2 of above (3) by hind part of latter part of α2 of above (1)	4

(Note 1) Reformed SP: MAVMAPRTLVLLLSGALTLTETWA

(Note 2) SP: signal peptide, α1: α1 domain, α2: α2 domain, α3: α3 domain, TM: transmembrane domain

As known from the results in Table 1, it is confirmed that the HLA-E chimeric molecule is efficiently expressed on the CHO cell, by replacing the SP sequence of HLA-E molecule by reformed SP (sequence number 21) similar to SP of HLA-G1, and replacing α2 domain (amino acid number 91-182) of HLA-E molecule, latter part (amino acid number 137-182) of α2

domain of HLA-E molecule, or fore part (amino acid number 137-150) of latter part of α2 domain of HLA-E molecule respectively by amino acid sequence corresponding to HLA-G1 molecule.

Example 2

Expression of various HLA-E chimeric molecules in nonhuman mammal cell (2)

A base sequence for coding the amino acid sequence composed as shown in Table 2 was incorporated into an expression vector of pCXN. Each transgene was incorporated into CHO cell, and FACS analysis was conducted by using anti-HLA antibody. Results are shown in Table 2.

Table 2. Expression of various HLA-E chimeric molecules in nonhuman mammal cell

Symbol	Molecule composition	Peak of FACS
(1) Vector	Vector only	4.94
(2) HLA-E	HLA-E molecule	15.07
(3) HLA-E(V)	Replaced SP of above (2) by reformed SP	36.84
(4) HLA-E(147)	Replaced serine of amino acid 147 of α2 domain of above (2) by cysteine	121.84
(5) HLA-E(V×147)	Replaced SP of above (4) by reformed SP	318.97

As known from the results in Table 2, it is confirmed that the HLA-E chimeric molecule replacing serine of amino acid number 147 of α2 domain of HLA-E molecule by cysteine of amino acid number 147 of α2 domain of HLA-G1 molecule is efficiently expressed on the CHO cell, and it is also confirmed that the HLA-E chimeric molecule replacing SP sequence of HLA-E molecule by reformed SP (sequence number 21) and replacing the amino acid sequence number 147 of α2 domain of HLA-E molecule by

cysteine is more efficiently expressed on the CHO cell. It is further confirmed that the HLA-E chimeric molecule can be more efficiently expressed on the CHO cell by replacing the hydroxyl group (-OH) of β-position of serine by thiol group (-SH) of cysteine.

Example 3

Resistance of nonhuman mammal cell expressing HLA-E chimeric molecule to cytotoxicity by human NK cell

A base sequence for coding the amino acid sequence composed as shown in Table 3 was incorporated into an expression vector of pCXN. Each transgene was incorporated into swine endothelial cell (SEC), and stable cell lines were prepared. Human NK-like cells (YT) were applied to transformed SEC cells at a rate of 5:1 (37°C, 4 hours), and lactate dehydrogenase (LDH) released from SEC was detected as index, and the cytotoxicity by human NK cell was measured, and relative value of cytotoxicity was determined. Results are shown in Table 3.

Table 3. Resistance of swine endothelia cells to cytotoxicity by human NK cell

Transformed cell	Description	Cytotoxicity (relative)
(1) SEC	No gene transduction operation	100[a]
(2) Mock	Transduction of Mock gene	94[a]
(3) HLA-E(V)	Transformation by base sequence of coding HLA-E chimeric molecule replacing SP of HLA-E molecule by reformed SP	85[b]
(4) HLA-E(V,147)	Transformation by base sequence of coding HLA-E chimeric molecule replacing SP of HLA-E molecule by reformed SP, and replacing amino acid 147 of α2 domain of HLA-E molecule by cysteine	35[c]
(5) HLA-E(V,11,147)	Transformation by base sequence of coding HLA-E chimeric molecule replacing amino acid 11 of α1 domain by alanine, in addition to above (4)	21[d]

(Note) a, b, c, d: There is significant difference among groups identified with different superscripts ($P<0.05$).

As known from the results in Table 3, it is confirmed that cytotoxicity by human NK cell can be suppressed in swine endothelia cell transformed by base sequence for coding any one of HLA-E chimeric molecule replacing SP of HLA-E molecule by reformed SP; HLA-E chimeric molecule replacing SP of HLA-E molecule by reformed SP and replacing amino acid number 147 of α2 domain of HLA-E molecule by cysteine; and HLA-E chimeric molecule replacing amino acid number 11 of α1 domain by alanine in addition to the above.